THE LOCALIZATION OF HEPARIN-LIKE BLOOD ANTICOAGULANT SUBSTANCES IN THE TISSUES OF SPISULA SOLIDISSIMA

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As previously reported (Thomas, 1951) a potent blood anticoagulant resembling heparin can be extracted from the common surf clam *Spisula (Mactra) solidissima.* This has recently been confirmed by Frommhagen *et al.* (1953). They attempted to develop the Spisula anticoagulant for clinical use.

The discovery of the Spisula anticoagulant stemmed from previous studies by Heilbrunn and his students concerning the biological significance of heparin and related substances. There is an increasing amount of evidence that such substances may be of rather general importance. Thus in the monograph by Jorpes (1946) there are numerous references indicating that in addition to acting as a blood anticoagulant heparin may serve other functions. It is known, for example, that heparin will inhibit growth in tissue culture and that it can inhibit the action of various enzymes. Heparin is chemically related to the sulfated polysaccharides found intercellularly in the connective tissues and in mucus secretions so that functions served by these latter substances are also of interest when considering the significance of heparin-like substances. As will be discussed later there is evidence that chondroitin sulfate may be of importance in calcification.

As compared with the number of investigations concerning heparin and related substances in mammals the number of similar investigations on adult invertebrate animals have been relatively few. However there is evidence that these substances may be of major importance to the eggs of invertebrate animals. Thus it is known that the jelly coat of sea urchin eggs contains a highly sulfated polysaccharide capable of preventing the clotting of blood. The significance of this fact and the general importance of the jelly coat to fertilization have been discussed by Runnström (1952). Polysaccharide sulfate esters seem to be of significance for reactions in the protoplasm of egg cells as well as for reactions at the surface. Thus Heilbrunn and Wilson (1949) found that heparin seems to inhibit the protoplasmic gelations which normally occur during division of the Chaetopterus egg. Also, as shown by Kelly (1953), not only the jelly coat but also certain elements in the protoplasm of some marine eggs show the metachromatic staining reaction for polysaccharide sulfate esters. The metachromatic reaction is a shift in color caused by the polymerization of certain basic dyes such as toluidine blue (Michaelis, 1947) and is often produced when the dye combines with large negatively charged molecules. Heparin and other highly sulfated polysaccharides produce a very intense red metachromatic color with toluidine blue.

From the histological investigations of Kelly it was known that the eggs and ovaries of Spisula exhibit strong metachromatic staining and, as will be shown in this paper, a metachromatic blood anticoagulant substance can be isolated from

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Spisula eggs. However it was observed that other tissues of this clam also had a very high affinity for toluidine blue and that breis of the tissues showed an impressive metachromasia *in vitro*. Thus it seemed desirable to isolate the metachromatic substance in order to determine some of its properties. Also an attempt has been made to discover the origin of the substance within the tissues. From the work of Soda and Egami (1938) it was known that a heparin-like anticoagulant can be obtained from the mucous secretions of *Charonia lampas,* a marine gastropod. Thus it was suspected that the anticoagulant from Spisula might also be of mucous origin. In part this seems to be true. However, blood clotting assays made on extracts of various portions of the clam have revealed that in addition to an anticoagulant of mucous origin another anticoagulant substance is present in the tissues of Spisula. From histological examination it appears that this latter substance is an intercellular material possibly analogous to the chondroitin sulfate of mammalian connective tissues. As pointed out in the discussion there is a possibility that such substances may be of importance in calcification processes.

Isolation of the Spisula Anticoagulant by Means of a Heparin Extraction Procedure

The clams (Spisula) were obtained from commercial fishermen along the New Jersey coast and at Woods Hole. At first, anticoagulant preparations were made from thoroughly washed clam meat containing all the organs except the viscera and shell. The visceral mass was removed because of complications caused by the gonadal material. The presence of sperm rendered it difficult to extract the anticoagulant. This was probably due to the basic proteins of the sperm which are known to precipitate heparin-like substances. On the other hand the viscera from female clams did not seem to contain any anticoagulant other than that which could be ascribed to the ovaries and eggs.

After grinding the clam meat several times in a food chopper, extraction and purification of the anticoagulant were carried out according to the procedure for preparing beef lung heparin described by Homan and Lens (1948). Thus the chopped tissue was allowed to autolyze for 24 hours and then was extracted for an hour with a warm alkaline buffer containing half normal sodium hydroxide and enough ammonium sulfate to maintain a pH of 9 or 10. The supernate was then heated to 70° C.–80° C. to denature protein, filtered, and the crude anticoagulant was precipitated together with protein by acidification to pH 2–3. The precipitate after extraction with alcohol was digested with trypsin. Some impurities could be removed from the digest by adding ammonium carbonate, centrifuging, and then boiling at pH 7 followed by another centrifugation. The active material was then precipitated by two volumes of alcohol and subjected to a partition between water and neutralized phenol. This left the active material in the aqueous phase whereas most of the remaining proteinaceous impurities entered the phenol phase. The product was obtained from the aqueous phase by adding NaCl and two volumes of alcohol. Further impurities could be removed with lead acetate at pH 5 and the lead removed with an excess of sodium carbonate. The anticoagulant was then obtained as the sodium salt by precipitation with a large excess of acetic acid followed by neutralization in an alcohol-ether mixture.

Anticoagulant tests were performed by the thrombin method of Jaques and Charles (1941), with the modification that instead of beef blood citrated sheep plasma was used. The anticoagulant activities of all clam preparations were compared to commercial samples of sodium heparin (generously supplied by the Upjohn Co., Kalamazoo, Mich.) rated in U.S.P. units by the manufacturer. These values ranged from 120 to 156 U.S.P. units/mg.

In all, three anticoagulant preparations were made from eviscerated clams as described above. Following the stage of phenol partition, the average anticoagulant activity of these preparations was 40 heparin units per milligram. By further purifying one of these preparations with lead acetate and then forming the sodium salt, a product with an activity of 50 heparin units per milligram was obtained. The yield obtained from these initial preparations was about 11,000 units per kilogram of starting material. The preparations that were carried only through the stage of phenol partition gave weak positive biuret and ninhydrin reactions indicating traces of protein material, but the lead-purified product seemed to be protein-free. All samples gave a positive color reaction for hexosamine (Palmer *et al.,* 1937) and a barium precipitate after hydrolysis with HCl indicated the presence of ester sulfate. In view of later findings it would appear that both the yield and activity from these initial preparations were rather low. Some of the probable reasons for this are discussed below.

The most active product obtained thus far was a lead acetate-purified sodium salt of the anticoagulant from Spisula mantle tissue. It had an activity of 130 heparin units per milligram which is equivalent to the activity of mammalian sodium heparinate. The yield of this final product from one kilogram of mantle was about 19,000 units which is equal to the best yields reported for beef lung heparin (Kuizenga and Spaulding, 1943). However, as will be discussed below, even this yield apparently represents only a fraction of the total anticoagulant substance in the Spisula mantle tissue. The final product was a white powder readily soluble in water, which seemed to be free of lead and proteins. After acid hydrolysis it gave positive tests for reducing sugar, hexosamine and ester sulfate. But it was interesting to note that the hexosamine color and barium sulfate precipitate appeared from qualitative examination to be only one half to two thirds as great as the same reactions given by beef lung heparin of the same anticoagulant strength.

The mantle tissue anticoagulant discussed above was purified from an alkaline extract in the same manner as the previous preparations. But in an effort to extract as much anticoagulant as possible, the mantle tissue was homogenized in a Waring Blendor and allowed to autolyze for a relatively long period (48 hours). It was then subjected to prolonged extraction (6 hours) with the alkaline sodium hydroxide-ammonium sulfate mixture. However, even after this more thorough extraction it was found that a large amount of the anticoagulant substance remained in the tissue. After extraction, the residue was washed with several changes of water and then with alcohol and ether. When stained with toluidine blue the residue still showed a strong metachromatic color and, after digestion of the residue with trypsin, the metachromatic material appeared in solution. The digest was boiled and centrifuged, then tested for anticoagulant activity, whereupon it was discovered that the digested residue yielded as much anticoagulant activity as the crude product from the alkaline extract. The alkaline extract and the digested residue each yielded about 30,000 units per kilogram of original tissue, thus in-

dicating a total potential yield of 60,000 units or more per kilogram of mantle tissue. It would seem, then, that alkaline extraction is not a very efficient method of removing the anticoagulant from Spisula tissues, and in subsequent work tryptic digestion was used as the method of extraction.

Since alkaline extraction did not remove all of the anticoagulant from the tissue it seemed wise to investigate other aspects of the procedure. By assaying tryptic digests of fresh and autolyzed mantle tissue it was found that no change in the total yield of anticoagulant was produced by autolysis. However more anticoagulant could be extracted with alkali after autolysis and the crude product obtained in this way was more active than when autolysis was omitted. This increased activity was probably due to the preferential destruction of contaminating substances during autolysis. Another factor of importance in obtaining a high final yield is that the active material precipitates rather slowly out of alcoholic solution after the removal of proteins. Even after the addition of salt to the alcoholic solution it was usually necessary to let the mixture stand for 24 hours or more. Centrifugation prior to this time often left some of the active material dispersed in the supernate. Partition of the crude product between neutralized phenol and water was a very effective step in purification if repeated two or three times. Provided that there was a clean separation of the two phases, none of the active material was found in the phenol phase. Most of the protein impurities following tryptic digestion could be removed in this manner.

Localization of Anticoagulant Substances Within the Tissues of Spisula

Extracts obtained from various organs and tissues were assayed for anticoagulant activity and these data were compared with results obtained from metachromatic staining of tissue sections and from *in vitro* observations on metachromasia.

For the histological localization of acid polysaccharides in the tissues, paraffin sections of formalin-fixed material were stained according to the method of Sylvén as described by Glick (1949). Staining was done with toluidine blue in 30 per cent alcohol followed by destaining in 95 per cent alcohol. For the assay of total anticoagulant content of various organs and tissues the following method was adopted. A small quantity of tissue was extracted with alcohol and ether to remove lipids, and the extracted tissue was then digested with trypsin (Difco 1 : 250). Usually an amount of trypsin equivalent to one tenth the extracted dry weight of the tissue was used. After 24 hours digestion (35° C., pH 8) the digest was boiled and insoluble material centrifuged down. The supernate, made to a known volume and representing a known weight of tissue, was then assayed for anticoagulant activity. Table I represents the average anticoagulant activities per wet weight of tissue for two series of digests made in the above manner. It seems reasonably certain that these values represent activity due only to the heparin-like substances in the tissue. Preliminary trials indicated that the anticoagulant action of digests of this type could be abolished entirely by adding toluidine blue. Furthermore, no loss of activity resulted from dialysis, phenol partition, or from precipitation with two volumes of alcohol if these steps were carried out carefully. It was also found that such digests had no effect on the clotting time of a purified fibrinogen-thrombin clotting system. This indicates that a co-factor is probably necessary for the action of the anticoagulant from Spisula as is known to be the case for heparin (Chargaff, Ziff and Moore, 1941).

As can be seen from Table I the eggs of Spisula appear to contain a relatively large amount of anticoagulant substance. A partially purified sample of this material was obtained from a tryptic digest of eggs previously extracted with alcohol and ether to remove lipids. After removal of some of the impurities by phenol partition, the active material was precipitated from the aqueous phase with alcohol. The material so obtained had an anticoagulant activity of about 20 heparin units per milligram. A large part of this anticoagulant from the egg no doubt was derived from the jelly coat, but some may also have been derived from elements in the protoplasm. As discussed below, not only does the jelly coat stain metachromatically but also there are regions in the interior of the egg that give a metachromatic color with toluidine blue. At the time the assays listed in Table I were performed, attempts to obtain anticoagulant jelly coat solutions by the acid sea water treatment of Vasseur (1947) proved unsuccessful. However this problem is being re-investigated and it now seems probable that the eggs used at that time, although fertilizable, were immature. Recent preliminary results indicate that although a thin jelly coat is present on immature Spisula eggs it is not easily removed by acid sea water,

TABLE I

Anticoagulant activity of various Spisula tissues

	Heparin units per gram tissue wet weight
Mantle edge inner fold	180
Mantle edge outer fold	130
Gills	140
Palps	160
Eggs	100
"Skin" of foot	45
Foot devoid of "skin"	30
Adductor muscle	20

but can be removed by treating the eggs with 3% NaCl containing 0.1 *M* Versene (generously supplied by the Bersworth Chemical Co., Framingham, Mass.) at pH 8. On the other hand, some of the jelly coat from ripe eggs seems to be rather easily removed by acidified (pH 3.5–4) sea water.

Recently, Spisula eggs [2] fixed in Zenker-formal fluid and sectioned at three microns have been stained with toluidine blue (0.1% toluidine blue in 30% alcohol with destaining in 95% alcohol) after extraction with hot 4 per cent trichloroacetic acid (TCA) according to the method of Monné and Hårde (1951). Presumably this extraction removes the nucleic acids. The jelly coat of both the extracted and unextracted sectioned eggs exhibited brilliant red metachromatic staining. Unextracted eggs showed an intense blue to purple color in the cytoplasm and a purple metachromasia in the nucleolus. The main bulk of the germinal vesicle was practically unstained. After TCA extraction the cytoplasmic staining was reduced to a pale blue except for a diffuse red metachromasia in the outermost region of the cortex. The cortical granules which had previously been obscured by the strong cytoplasmic staining were very prominent after TCA extraction. These were seen to stain with an intense blue color. The staining of the nucleolus was not changed

[2] I wish to thank Dr. W. S. Vincent, Department of Anatomy, Syracuse Medical Center, Syracuse, New York, for the sectioned eggs.

appreciably by TCA extraction except that the metachromatic red color was more prominent. A more thorough study of the Spisula egg is planned and will be reported at a later date. It would be particularly interesting to determine more precisely whether the metachromatic staining of the nucleolus and cortical region is due to the presence of sulfated polysaccharides. In a preliminary report Allen (1951) mentions that mucopolysaccharide (as determined by the method of Monné and Slautterbach, 1950) appears to be transferred from the nucleolinus to the spindle during cleavage of the Spisula egg.

Returning now to a discussion of the adult clam, it will be observed (Table I) that digests of the mantle, gills and palps had five or six times more anticoagulant activity than digests of the foot and muscles. Histological sections of the mantle edge revealed several regions of metachromasia. The mucous cells of the inner and outer folds (adjacent to the shell) of the mantle edge are both metachromatic. However it was observed that the mucous cells of these two folds are distinctly different with respect to intensity of staining reaction and with respect to size and shape. Thus the mucous cells of the inner mantle surface are smaller and take the metachromatic color much more intensely than the mucous cells adjacent to the shell. No mucous cells were observed on the middle fold of the mantle edge. The mucosal basement membranes were stained a brilliant red as were certain areas of connective tissue in the interior of the mantle. Distally in the mantle folds this connective tissue is a dense compact material resembling cartilage. The histological picture for the gills and palps resembles that of the mantle. Thus the basement membranes and interior connective tissue of these structures also are metachromatic. The mucous cells lining the palps and gill filaments resemble the mucous cells lining the inner surface of the mantle. Probably these mucous cells, peculiar to the mantle cavity, provide the sticky secretion which aids in the collection of food particles. The secretion of the large mucous cells of the outer mantle fold possibly enters into the composition of the shell matrix.

Very little metachromasia was observed in cross sections of the foot and none could be detected in the muscle tissues. The foot was seen to contain large mucous cells in the perimeter. These were stained a pronounced blue color with toluidine blue but little, if any, of the metachromatic red color was evident. The mucosal basement membrane in the foot, however, stained metachromatically.

The known presence of a heparin-like substance in gastropod mucus suggested that the anticoagulant from Spisula might be of mucous origin. Also the intense metachromasia exhibited by the mucous cells lining the gills, palps and inner mantle surface suggested that the mucus from these cells might contain a highly sulfated heparin-like polysaccharide. However the intensity of the metachromatic staining observed in the connective tissues of the mantle and elsewhere suggested that there might be another heparin-like substance in the connective tissues. In order to determine this, mucosal scrapings were taken from both sides of the mantle edge and the three folds of the mantle edge were separated from one another. During these operations special precautions were taken to insure that no mucous contamination was transferred from one part of the mantle edge to another. After extraction of the tissue and mucus fractions with alcohol and ether, tryptic digests were made of these fractions, as described previously, and the digests were assayed for anticoagulant activity. In Table II are given the results of these assays together with the dry weights of the starting material after extraction of lipids. The numbers at the far

left of the table identify the different types of data obtained from a given series of digests. To obtain the metachromatic ratios given in Table II a series of tubes, each containing the same amount of toluidine blue with phosphate buffer (pH 6.6) plus increasing concentrations of digest, was matched in a comparator block with an identical series of tubes containing buffered toluidine blue and heparin. The ratios are the number of anticoagulant units of heparin required to produce a given color, divided by the number of anticoagulant units of digest required to produce the same color. Thus, for example, if a digest had a metachromatic ratio of three, only one-third of an anticoagulant unit of digest would be required to produce the same mixture of red and blue color with a given amount of toluidine blue as was produced by one anticoagulant unit of heparin.

TABLE II

Anticoagulant activities and metachromatic ratios for various portions of the mantle edge

Inside mantle edge mucosa	Outside mantle edge mucosa	Remainder of mantle edge after scraping			
		Whole mantle edge	Inside mantle fold	Outside mantle fold	Middle mantle fold
A. Heparin units per mg. dry weight					
1) 1.44	0.28	0.80			
2) 1.30	0.07	0.68			
3) 0.98	0.08		0.84	0.64	
4)			0.80	0.72	0.93
B. Total weight of mantle edge and mucosal scrapings in milligrams					
1) 44	60	2,480			
2) 32	48	1,700			
C. Metachromatic ratio of extracts (see text)					
1) 5.6	not meta-	0.8			
2) 5.2	chromatic	1.0			
3) 4.4			1.6	1.0	1.2

It will be noted that the tryptic digests of mucus from the outside mantle fold had little if any anticoagulant activity. Although this mucus was metachromatic in tissue sections, tryptic digests of this mucus were not metachromatic. On the other hand, the digested mucus from the inner mantle fold had anticoagulant activity and was highly metachromatic. In fact, it was about five times as metachromatic per anticoagulant unit as the heparin standard. Apparently this mucous substance has the ability to bind toluidine blue very strongly in comparison with its ability to prevent blood clotting. In digests of mantle edge tissue after scraping off the mucosas the metachromasia was about equal to that of heparin.

The data in Table II clearly indicate that another anticoagulant factor is present in the mantle edge tissue besides that originating from the mucous secretions. In the first place, digests of the outer mantle fold had nearly as much anticoagulant activity as those of the inner mantle fold, in spite of the fact that the mucus of the outer fold

was nearly inactive. Quite possibly the slight activity that was associated with this mucus can be ascribed to the small amount of tissue scraped off with the mucus. It will be observed that the middle fold had a high anticoagulant activity but, as stated previously, the middle fold of the Spisula mantle edge is apparently devoid of mucous cells. It is true that the highest anticoagulant activity per weight of starting material was given by the mucosal scrapings from the inside mantle fold. However, these scrapings had less than twice the potency of the scraped mantle edge and weighed only about one fiftieth as much. Furthermore, histological examination of scraped mantle edges showed that more than half of the mucosa had been removed. This means then that only about one twenty-fifth of the total anticoagulant activity of the mantle edge can be accounted for by the inner mucosa. Thus the only other obvious source of heparin-like anticoagulant in the Spisula mantle edge is the strongly metachromatic substance in the connective tissue.

Since the middle fold of the mantle is very rich in metachromatic connective tissue substance but has few if any mucous cells, it seemed desirable to isolate a sample of anticoagulant exclusively from this portion of the mantle. The middle fold was cut from mantle edge of several clams and thoroughly cleaned of debris.

TABLE III

Anticoagulant from middle fold of Spisula mantle edge

Tissue wgt., milligrams		Product wgt., milligrams	Heparin units per milligram	
Wet	Dry		Product	Dry tissue
4,950	830	14	45	0.75

After alcohol-ether extraction and tryptic digestion of the tissue, the digest was boiled and centrifuged to remove impurities. Following this the active material was precipitated from solution with two volumes of alcohol and redissolved in water. This solution was then shaken out with two changes of phenol neutralized with ammonium hydroxide. After acidifying the aqueous supernate to pH 4 or 5, NaCl was added to a concentration of 1% and two volumes of alcohol were added. The solution was heated to 50° C. and allowed to stand for 24 hours, after which time the active precipitate adhered firmly to the vessel. This material was washed with an alcohol-ether mixture, then taken up in a small amount of water and allowed to dry in a weighing bottle over $CaCl_2$. After weighing, this material was made to a known concentration and assayed for anticoagulant activity. The results are given in Table III. Wet weight of the starting material refers to the fresh tissue and dry weight refers to this tissue after extraction with alcohol and ether. The heparin units per milligram dry tissue refer to the amount of anticoagulant recovered in the product. The final product (Table III) was quite metachromatic and as can be seen its anticoagulant activity was almost half that of genuine heparin. Undoubtedly further purification would increase the activity. The amount of anticoagulant recovered per milligram of dry starting material is nearly as much as was indicated from the assay of crude digests in Table II.

Discussion

Probably the anticoagulant substance from the middle fold of Spisula mantle is identical with the metachromatic substance detected histologically in the connective tissues. This substance, although most abundant in the mantle and associated structures, is apparently present in other parts of the clam. Thus the basement membrane in the foot mucosa was quite metachromatic. No metachromasia was detected histologically in certain other tissues such as the adductor muscle, but digests of adductor muscle were slightly metachromatic corresponding to the low anticoagulant activity of these digests.

It seems possible that this connective tissue substance serves much the same function in the clam as does chondroitin sulfate in the tissues of mammals. Thus it is interesting that chondroitin sulfate has been implicated in calcification processes. Neuman and co-workers (1952) have shown that the chondroitin sulfate in cartilage acts as a cation exchange resin. Also Miller, Waldman and McLean (1952) found that toluidine blue and other basic dyes which have a high affinity for polysaccharide sulfate esters can prevent the *in vitro* calcification of hypertrophic cartilage. Apparently this inhibition is reversible. As they point out, there are various interesting correlations between metachromatic staining coincident with calcification. Thus Rubin and Howard (1950), for example, found that the metachromatic staining of growing bones is most intense in those regions about to calcify.

In view of the apparent relationship between calcification and acid polysaccharides in mammalian tissues, the recent paper by Bevelander (1952) was read with considerable interest. From his radioautograph showing distribution of Ca^{45} in the mantle edge of Anodonta it would appear that calcium is taken up in certain regions of the mantle edge connective tissues. Similar regions in the Spisula mantle edge appear to contain an abundance of acid polysaccharide. A further investigation is planned to determine in what way mucopolysaccharides could be involved in the calcification of molluscs.

I wish to express my sincere gratitude to Dr. L. V. Heilbrunn for his encouragement and advice during this investigation.

Summary

1. A heparin-like blood anticoagulant has been isolated from the surf clam *Spisula solidissima*.

2. The most potent preparation obtained had an anticoagulant activity of 130 U.S.P. heparin units per milligram and was derived from mantle tissue.

3. Anticoagulant assays made on tryptic digests from various portions of the clam revealed that the mantle, gills and palps had about five times more anticoagulant activity per gram of tissue than the foot and adductor muscles. It was also found that the eggs of Spisula yield a high anticoagulant activity.

4. Toluidine blue staining of sectioned eggs revealed metachromasia in the jelly coat, cortical region and nucleolus.

5. In order to determine the origin of the anticoagulant from the adult clam, the results from anticoagulant assays made on digests of isolated portions of the mantle edge were compared with histologic observations concerning metachromasia. It was concluded that at least two substances with heparin activity are present in the mantle tissue. One of these substances is present in the mucus secretion of

the inner mantle fold and apparently also in the mucus secretions of the palps and gills. The mucus secretion of the outer mantle fold was nearly devoid of anticoagulant activity. The other anticoagulant substance seems to be an intercellular material in the connective tissues, possibly analogous to chondroitin sulfate. This substance also seems to be most abundant in the mantle, palps and gills.

6. The possibility that polysaccharide sulfate esters may be important in calcification processes is discussed.

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